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Role of methylene blue and chromatin proteins in the formation of DNA-protein crosslinking induced by visible light

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Abstract : DNA-protein crosslinking (DPC) was studied in chromatin isolated from sarcoma-180 ascitic tumour cells under the action of visible light in presence of methylene blue (MB), a photosensitive dye. For crosslinking experiments, sonicated chromatin was kept in 10 mM Tris-HCl buffer. MB-chromatin samples were irradiated with a Philips 160W high pressure mercury lamp (major range of emission=540-740 nm). Amount of DNA crosslinked with protein was quantified by measuring the absorption at 260 nm of the aqueous phase obtained from the sodium dodecyl sulfate-chloroform-isoamyl alcohol extraction assay. It was observed that the amount of DPC produced varied in a dose dependent manner with a maximum obtained at dye-DNA nucleotide ratio of 0.04 and 60 minute irradiation. MB acted as a mediator in the crosslinking phenomenon. For identification of proteins involved in crosslinking to DNA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with 15% separating gel for both the control and treated samples. Coomassie blue stained gels were scanned at 560 nm. Almost all the nonhistone protein bands were found to be absent from the gel pattern of the treated sample, whereas a reduction in the amount of histone protein was observed. Role of intercalative binding of MB in DPC formation was tested by a competition experiment with ethidium bromide (EB) which is known to compete with MB for the intercalation sites in DNA. Competition with EB in MB-chromatin sample reduced crosslinking in a dose dependent manner indicating that intercalation of MB with DNA of chromatin is essential for crosslink formation.

Keywords : DNA-protein crosslink, chromatin, methylene blue, visible light.

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1. Introduction

In eukaryotic systems, chromatin plays a central role in metabolic processes such as replication, transcription and translation to form chromatin. Most of the chromatin proteins can be dissociated from DNA simply by treatment with high salt and/or detergent (Ohlenbusch *et al* 1967, Strniste and Rall 1976). If by some physical or chemical means the association of such proteins with DNA is made stable to the above treatments, the phenomenon is known as DNA-protein

crosslinking (Smith 1962). In the present work, formation of DPC by the action of visible light in presence of MB has been studied. MB is a photo-sensitive intercalating dye. Sarcoma-180 ascites tumour cells are used as a source for chromatin.

2. Materials and methods

2.1. Chemicals :

MB, EB, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), Triton X-100 were purchased from Sigma Chemical Co (USA). All other chemicals were of analytical grade.

2.2. Optical measurements :

All the solutions were prepared in 10 mM Tris-HCl buffer (pH 7.4) (at 25°C). Absorbance studies were performed (cuvette path length 1.0 cm) in a Pye-Unicam SP8-100 UV-visible spectrophotometer at room temperature (25°C).

2.3. Isolation of chromatin :

The nuclei were isolated from 7-9 day old sarcoma-180 tumour cells (Karuri and Mukherji 1988). Chromatin was isolated from nuclei by lysing in 1 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM PMSF, washed and stored at −2°C in the above mentioned buffer containing 12.5% glycerol. All experiments were done with chromatin within one week after preparation.

On the day of experiment, chromatin was suspended in 10 mM Tris-HCl, pH 7.4, sonicated for 15 secs in a Labsonic 2000 sonicator at a 50W setting and centrifuged at 1000 g to clear the aggregates. The supernatant was used for crosslinking experiments.

2.4. Concentration measurements :

The DNA content of chromatin was determined from spectrophotometric absorption using $\epsilon_{260} = 7260$ (M^{-1} cm^{-1}) (Smart and Bonner 1971). Protein concentration was determined by the Lowry method (Lowry *et al* 1951) with BSA as the standard. The protein to DNA ratio of chromatin was found to be 1.65 ± 0.05. The concentration of MB was determined using $\epsilon_{665} = 96{,}000$ (Sastry and Gordon 1966) and the concentration of EB was determined using $\epsilon_{480} = 5600$ (Waring 1965).

2.5. Light irradiation :

Chromatin samples kept over crushed ice were irradiated with visible light using a Philips MLL-N 160W mercury lamp with a maximum in the range 540-740 nm. The light was filtered through a 1.0 cm water layer and 0.5 cm glass to absorb heat and possible UV. Radiation dose rate was 1.6×10^5 ergs/cm^2/sec.

2.6. SDS-chloroform-isoamyl alcohol (SDS-CIA) assay :

This assay was a slight modification of the method of Mandel et al (1979). Samples were adjusted to 0.2% SDS, 1.0 M NaCl, kept at 60°C for 10 mins, vortexed with an equal volume of chloroform-isoamyl alcohol (24 : 1) and centrifuged for 5 mins at 1000 g to clearly separate the phases. The decrease in absorbance at 260 nm (A_{260}) of the aqueous phase at irradiation time t with respect to control (0 min) gave an estimation of the extent of DNA crosslinked with protein.

$$\text{Percentage DNA crosslinked} = \frac{A_{260}(0) - A_{260}(t)}{A_{260}(0)} \times 100$$

2.7. Polyacrylamide gel electrophoresis :

Chromatin proteins were analysed on a 15% SDS-polyacrylamide slab gel using the procedure of Laemmli (1970) with a 4.5% stacking gel. Chromatin samples were

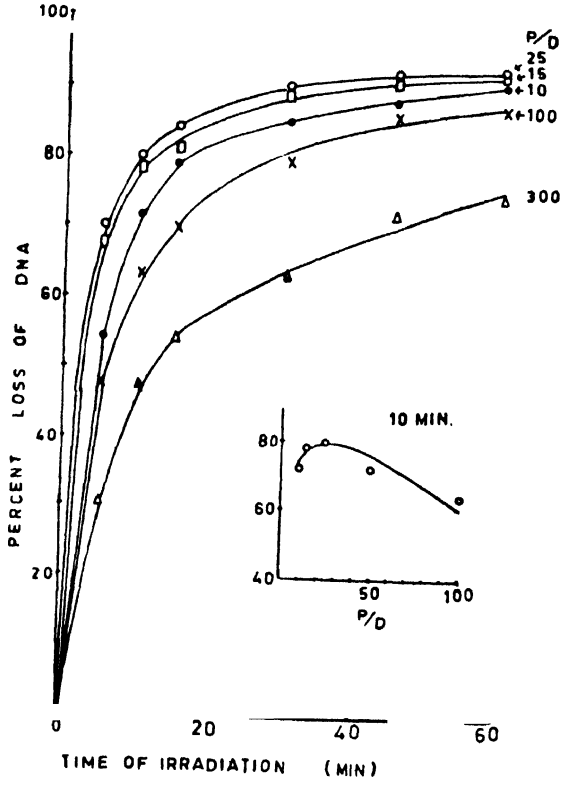

Figure 1. Kinetics of DPC formation in MB-chromatin at various D/P ratios. MB-chromatin samples (DNA nucleotide concentration, 1.5×10^{-4}M) were prepared at different D/P ratios, kept in separate Petri dishes and irradiated. Aliquots were processed by SDS-CIA assay. Inset: percentage DNA crosslinked at 10 min of irradiation for different D/P ratios.

concentrated by dialysing against dry sucrose at 4°C. It was made 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol and 0.001% bromophenol blue and boiled for 3 mins. 25-30 μg DNA of chromatin was loaded to each lane and electrophoresed. Gels were stained with 0.1% Coomassie brilliant blue R and subsequently destained with 5% methanol, 10% acetic acid. Gels were scanned at 560 nm in a SP8-100 Pye-Unicam Spectrophotometer.

3. Results and discussions

Figure 1 shows the percentage DNA crosslinked of MB-chromatin at increasing doses of radiation for different dye-DNA nucleotide (D/P) ratios by SDS-CIA assay.

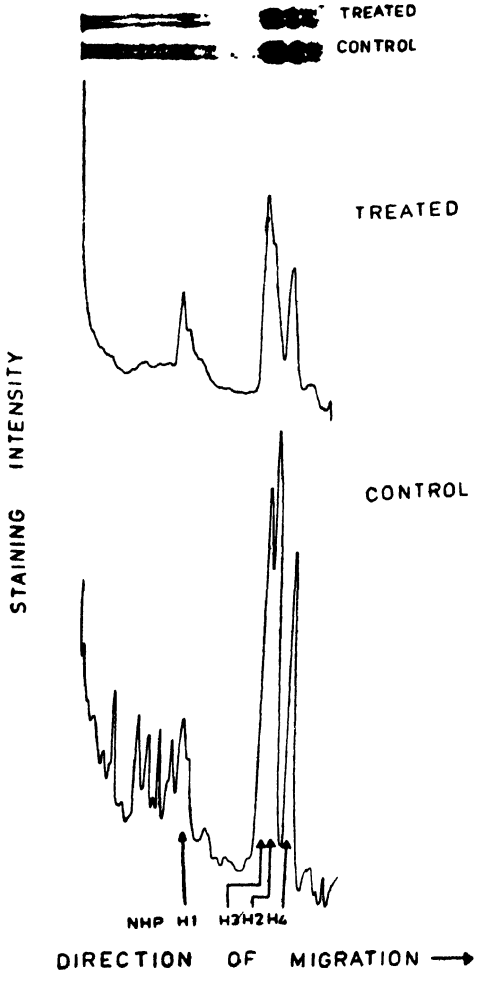

Figure 2. Photograph of Coomassie blue stained SDS-polyacrylamide gel of chromatin proteins (upper panel) and their densitometric tracings at 560 nm (lower panel). Control and treated (60 min irradiated) MB-chromatin at a D/P ratio of 0.04 were processed for electrophoresis.

The rate of crosslink formation was very rapid initially and gradually slowed down, finally reaching a plateau. 92% DNA was found to be lost from the aqueous phase at $D/P=0.04$, 60 min radiation. A lowering in dye concentration, even $D/P=0.0033$, produced 75% DNA crosslinked under 60 min light exposure. Since the formation of DPC was decreased by a decrease in dye concentration, it was

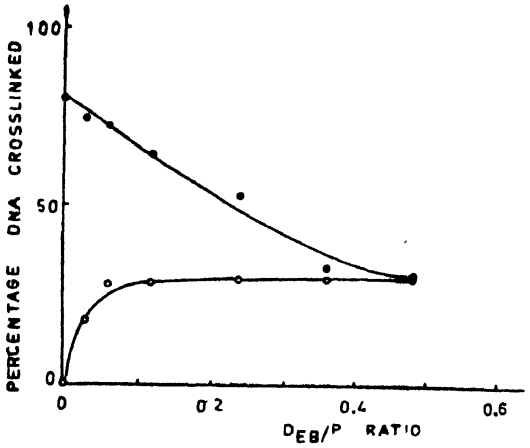

Figure 3. Effect of EB on DPC formation in MB-chromatin. EB was added to chromatin (DNA nucleotide concentration, 1.5×10^{-4}M) with ($D_{MB}/P=0.04$) or without MB. These samples were irradiated separately for 30 min and processed by SDS-CIA assay ; one aliquot of each without irradiation served as a control. EB-chromatin (○—○) ; EB-MB-chromatin (●—●).

studied for a number of D/P ratios. However, there was an apparent peak at $D/P=0.04$ (Figure 1, inset). That MB acted as a mediator in the crosslinking phenomenon was verified by Sepharose 4B-CL column chromatography (data not shown).

For the identification of proteins crosslinked to DNA, SDS-polyacrylamide gel electrophoresis was the method of choice. Chromatin proteins get resolved in this gel but high molecular weight DNA and also crosslinked proteins do not enter the gel. Therefore, a decrease in the staining intensity of a band belonging to a particular protein with respect to control, indicates its crosslinking to DNA. Figure 2 shows a photograph of chromatin gels and their densitometric tracings. Compared to control, most of the nonhistone proteins did not enter the gel in the case of light treated sample. Among the histones, H2 was maximally crosslinked while H1 was affected least.

To explore the necessity of intercalation of MB in DPC formation, a competition experiment was set up using EB. It is known that EB competes with MB for the intercalation sites in DNA (Lee *et al* 1973). From the lower curve in Figure 3, it can be seen that like MB, EB can induce DPC formation during visible light exposure though the extent of crosslinking is much less. As shown in the upper

curve of Figure 3, the presence of EB in MB-chromatin reduced crosslinking in a dose dependent manner which ultimately masked the contribution of MB($D_{EB}/P = 0.48$). This is possibly due to the reduction of binding of MB to chromatin by competition with EB (data not shown).

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